



# Phylogenetic Analyses Reveal a Unique Species of *Elaphe* (Serpentes, Colubridae) New to Science

LING Chen<sup>1,\*</sup>, LIU Shaoying<sup>2,\*</sup>, HUANG Song<sup>1,\*\*</sup>, Frank T. Burbrink<sup>3</sup>, GUO Peng<sup>4</sup>, SUN Zhiyu<sup>2</sup> and ZHAO Jie<sup>2</sup>

<sup>1</sup> College of Life and Environment Sciences, Huangshan University, Huangshan 245021, Anhui, China

<sup>2</sup> Sichuan Academy of Forestry, Chengdu 610066, Sichuan, China

<sup>3</sup> Department of Biology, College of Staten Island, The City University of New York, 2800 Victory Blvd, Staten Island, NY 10314, USA

<sup>4</sup> Department of Life Sciences and Food Engineering, Yibin University, Yibin 644000, Sichuan, China

**Abstract** The snakes comprising the monophyletic group referred to as ratsnakes are found throughout Asia, Europe and the New World. Recently, three snake samples likely belonging to the ratsnakes were collected in Zoige County, Sichuan Province, China. Species identity was difficult to delimit morphologically because the specimens were juveniles and partially damaged. Subsequently, a molecular phylogenetic approach was used. Portions of three mitochondrial genes (cyt *b*, ND4 and 12S rRNA) were sequenced and analyzed. The results showed that they were sister to the genus *Elaphe*. Very little genetic variation was found among the three samples. The minimum genetic distances between these samples and those within *Elaphe* were greater than any currently recognized species within the genus. We conclude that this likely represents a new species within the genus *Elaphe*. Adult specimens and a morphologic description are needed for further study.

**Keywords** *Elaphe*, ratsnake, phylogenetic analysis, mitochondrial gene, genetic distance

## 1. Introduction

The systematics of the group referred to as ratsnakes have been studied for more than a century but recently this group has been restricted to include the Old World genera *Coelognathus*, *Gonyosoma*, *Rhadinophis*, *Maculophis*, *Euprepiophis*, *Oreophis*, *Orthriophis*, *Elaphe*, *Zamenis*, *Rhinechis*, *Oocatochus* and *Coronella* as well as 9 genera in the New World tribe Lampropeltini (Utiger *et al.*, 2002; Burbrink and Lawson, 2007). The ratsnakes likely originated in the mid-Eocene in southeastern Asia, currently occurs throughout Asia, Europe and the New World, and is composed of 72 species (Schulz, 1996; Burbrink and Lawson, 2007; Burbrink and Pyron, 2010;

www.reptile-database.org). Within China, there are 15 known species belonging to six genera (species numbers in parenthesis): *Elaphe* (6), *Rhadinophis* (2), *Orthriophis* (3), *Euprepiophis* (2), *Oreophis* (1), and *Coelognathus* (1) (Zhao, 2006). However, it is likely that given the large area of this country, with differed landforms and diverse habitats, that more taxa have evaded discovery. For instance, the Zoige Grassland in northwestern Sichuan is located within the northeastern part of the Tibetan Plateau. In this region, only two snake species have been documented: *Gloydus strauchi* and *Elaphe dione* (Zhao, 2003). Furthermore, it is likely that given the general cryptic nature of snakes that more species occur in this area (Ursenbacher *et al.*, 2006; Huang *et al.*, 2009).

In August 2008, two neonate snakes (NS1, NS2) and one juvenile snake (JS) were collected in Zoige County (specific sites unknown) by local farmers. The juvenile was found dead on road (DOR) and the forepart of its body was crushed, making its positive identification impossible. Furthermore, identifying the exact species and

\* Both authors contributed equally to this work.

\*\* Corresponding author: Prof. HUANG Song, from the College of Life and Environment Sciences, Huangshan University, with his research focusing on ophiology, phylogenetics and biogeography of reptiles.

E-mail: snakeman@hsu.edu.cn

Received: 21 June 2010 Accepted: 12 November 2010

genus of these three specimens was difficult based on the available material. Fortunately, muscle tissue was preserved in 95% ethanol, which permitted molecular phylogenetic analyses to be conducted in order to identify the genus of the specimens.

Over the last two decades, the analyses of mitochondrial DNA genes have successfully been used as a versatile and effective tool to investigate the phylogenetic relationships of snakes (Huang *et al.* 2009). The most frequently used fragments are mitochondrial cytochrome *b* (cyt *b*) and/or NADH dehydrogenase subunit 4 (ND4) genes (e.g. Kraus and Brown, 1998; Kelly *et al.*, 2003; Malhotra and Thorpe, 2004; Lawson *et al.*, 2005; Burbrink and Lawson, 2007). Using these gene fragments, Huang *et al.* (2009) successfully found the closest related living relatives of the hot-spring snakes (Colubridae, *Thermophilis*), the relict species endemic to the Tibetan Plateau.

This study aims to first determine which subfamily these three samples would belong to within Caenophidia by using the datasets of Huang *et al.* (2009); and then with the result, to further examine the relationships within the subfamily by using more samples from that group. The results from this study suggest that the samples are most likely allied to the ratsnakes, particularly the genus *Elaphe*.

## 2. Material and methods

**2.1 Dataset construction** The specimens (NS1, NS2, and JS) are deposited in the Museum of the Sichuan Academy of Forestry (field numbers ZJ200801-03). Total genomic DNA was extracted with phenol/chloroform as described in Sambrook *et al.* (1989). Complete cytochrome *b* (cyt *b*), partial ND4, and partial 12S rRNA genes were amplified from the total DNA extracts using the polymerase chain reaction (PCR) with primer pairs L14910/H16064 (Burbrink *et al.*, 2000), ND4/Leu (Arévalo *et al.*, 1994), and L1091mod/H1557mod (Zaher *et al.*, 2009), respectively. The PCR reaction contained approximately 100 ng of template DNA, 1  $\mu$ L of each primer, 5  $\mu$ L of  $\times 10$  reaction buffer, 2  $\mu$ L dNTPs (each 2.5 mM), and 2.0 units of *Taq* DNA in a 50  $\mu$ L reaction. Reactions were cycled at the following temperatures: an initial 94°C denaturation for 3 min, 30 cycles of 94°C denaturation for 1 min, 46–55°C annealing for 1 min, 72°C extension for 1 min, and a final 72°C extension for 5 min. Genes were purified from 0.8% low-melting agarose gels using the BioStar Glassmilk DNA purification Kit ac-

cording to manufacturer's instructions. The purified DNA was sequenced with the Perkin-Elmer BigDye DNA Sequencing Kit according to the manufacturer's protocol with the primers used in PCR.

To determine the phylogenetic position of the three samples, we used the three datasets from Huang *et al.* (2009) which contained cyt *b* sequences for 130 species, ND4 sequences for 112 species, and a combined cyt *b* and ND4 dataset for 85 species. From these datasets, we determined that the snakes were likely related to the ratsnakes. Therefore, we downloaded these genes for 34 species of ratsnakes from GenBank which were used to further determine the specific phylogenetic relationships of these unidentified samples (Table 1). Of the 10 species of *Elaphe* (The closest relatives of the three samples, see Results) available from GenBank, *E. davidi*, *E. sauro-mates* and *E. anomala* were not available for cyt *b* and ND4. However, we acquired 17 sequences of partial 12S rRNA, including all the 10 species of *Elaphe*, that is, 3 from *E. bimaculata*, 6 from *E. dione* and 1 from each of the rest 8 species. Additionally, 14 other genera of ratsnakes were downloaded from GenBank (Table 1).

**2.2 Phylogenetic inference** Sequences for cyt *b*, ND4 and 12S rRNA were aligned with other retrieved sequences for the same gene regions respectively, and to the combined gene sequences using Clustal X (Thompson *et al.*, 1997), and further proofread by eye. Bayesian inference (BI) and maximum likelihood (ML) approaches were used to infer phylogenies using MrBayes v3.0 (Huelsenbeck and Ronquist, 2001) and RAxML Black-Box (<http://phylobench.vital-it.ch/raxml-bb/index.php>; Stamatakis *et al.*, 2008). Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the model of nucleotide substitution under the Akaike Information Criterion (AIC) for ML and Bayesian Information Criterion (BIC) for BI. In the Bayesian analysis, a starting tree was obtained using the neighbor-joining algorithm. Posterior probabilities (PP) were obtained by Markov chain Monte Carlo (MCMC) analysis with one cold chain and three heated chains. Samples of trees and parameters were drawn every 100 steps from a total of 1,000,000 MCMC steps. The first 100,000 generations (1,000 trees) were discarded as burn in (representing the generations in which the chain had not yet reached stationarity) and only the results from the last 900,000 generations (9,000 trees) were used to compute a consensus tree. We ran three additional analyses starting with random trees. The consensus of all the post-burn generations (3,600,000 generations resulting in 36,000 trees) was estimated from all four runs.

**Table 1** Sequences of cyt *b*, ND4 and 12S rRNA genes from the ratsnakes and *Ptyas* (outgroup) retrieved from GenBank

| Genus and species                 | Accession No. |          |          | Sources                                                     |
|-----------------------------------|---------------|----------|----------|-------------------------------------------------------------|
|                                   | cyt <i>b</i>  | ND4      | 12S rRNA |                                                             |
| Old world                         |               |          |          |                                                             |
| <i>Coronella austriaca</i>        | AY486930      | AY487065 |          | Nagy <i>et al.</i> (2004)                                   |
| <i>Coronella girondica</i>        | AF471088      | AY487066 | AY122835 | Lawson <i>et al.</i> (2005); Utiger <i>et al.</i> (2002)    |
| <i>Elaphe anomala</i>             |               |          | AY122803 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe bimaculata</i>          | DQ902104      | DQ902283 |          | Burbrink and Lawson (2007)                                  |
| <i>Elaphe bimaculata</i>          |               |          | AY122837 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe bimaculata</i>          |               |          | AY122767 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe bimaculata</i>          |               |          | AY122768 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe carinata</i>            | DQ902133      | DQ902284 | AY122838 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Elaphe climacophora</i>        | DQ902105      | DQ902285 | AY122772 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Elaphe davidi</i>              |               |          | AY122775 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               | DQ902107      | DQ902287 |          | Burbrink and Lawson (2007)                                  |
| <i>Elaphe dione</i>               |               |          | AY122834 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               |               |          | AY122832 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               |               |          | AY122833 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               |               |          | AY122830 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               |               |          | AY122831 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe dione</i>               |               |          | AY122829 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elapheb quadrvirigata</i>      | DQ902120      | DQ902300 | AY122794 | Burbrink and Lawson (2007) ; Utiger <i>et al.</i> (2002)    |
| <i>Elaphe quatuorlineata</i>      | AY486931      | AY487067 | AY122796 | Nagy <i>et al.</i> (2004) ; Utiger <i>et al.</i> (2002)     |
| <i>Elaphe sauromates</i>          |               |          | AY122797 | Utiger <i>et al.</i> (2002)                                 |
| <i>Elaphe schrenckii</i>          | DQ902124      | DQ902302 | AY122804 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Euprepiphis conspicillatus</i> | DQ902106      | DQ902286 |          | Burbrink and Lawson (2007)                                  |
| <i>Euprepiphis mandarinus</i>     | DQ902115      | DQ902294 | AY122784 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Oocatochus rufodorsatus</i>    | DQ902123      | DQ902301 | AY122800 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Oreophis porphyraceus</i>      | DQ902118      | DQ902298 | AY122790 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Orthriophis cantoris</i>       | DQ902135      | DQ902315 |          | Burbrink and Lawson (2007)                                  |
| <i>Orthriophis hodgsonii</i>      | DQ902136      | DQ902318 |          | Burbrink and Lawson (2007)                                  |
| <i>Orthriophis moellendorffi</i>  | DQ902116      | DQ902295 |          | Burbrink and Lawson (2007)                                  |
| <i>Orthriophis taeniurus</i>      | EF076709      | DQ902305 | AY122809 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Rhinechis scalaris</i>         | AY486932      | AY487068 | AY122801 | Nagy <i>et al.</i> (2004); Utiger <i>et al.</i> (2002)      |
| <i>Zamenis hohenackeri</i>        | DQ902137      | DQ902320 | AY122779 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Zamenis situla</i>             | DQ902125      | DQ902303 |          | Burbrink and Lawson (2007)                                  |
| NS1 and NS2 (NSs)                 | HQ330524      | HQ330526 | HQ330522 | This study                                                  |
| JS                                | HQ330525      | HQ330527 | HQ330523 | This study                                                  |
| <i>Ptyas korros</i>               | AY486929      | AY487062 |          | Nagy <i>et al.</i> (2004)                                   |
| <i>Ptyas mucosus</i>              | AF471054      | AY487063 |          | Lawson <i>et al.</i> (2005)                                 |
| New world                         |               |          |          |                                                             |
| <i>Arizona elegans</i>            | DQ902101      | DQ902279 | AY122810 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Bogertophis subocularis</i>    | DQ902103      | DQ902281 |          | Burbrink and Lawson (2007)                                  |
| <i>Bogertophis rosaliae</i>       | DQ902102      | AF138751 | AY122815 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Lampropeltis getula</i>        | FJ997848      | AF138759 |          | Pyron <i>et al.</i> (2009)                                  |
| <i>Lampropeltis alterna</i>       | AF337130      | AY497307 |          | Fetzner <i>et al.</i> (Unpubl.)                             |
| <i>Lampropeltis calligaster</i>   | DQ902129      | DQ902311 |          | Burbrink and Lawson (2007)                                  |
| <i>Lampropeltis ruthveni</i>      |               |          | AY122820 | Utiger <i>et al.</i> (2002)                                 |
| <i>Pseudelaphe flavirufa</i>      | DQ902109      | DQ902289 | AY122841 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |
| <i>Pantherophis obsoletus</i>     | AF283644      | DQ902296 | AY122843 | Burbrink <i>et al.</i> (2000) ; Utiger <i>et al.</i> (2002) |
| <i>Pantherophis vulpinus</i>      | AF283638      | AF138758 |          | Burbrink <i>et al.</i> (2000)                               |
| <i>Pantherophis bairdi</i>        | AF283599      | AF138755 |          | Burbrink <i>et al.</i> (2000)                               |
| <i>Pantherophis guttatus</i>      | DQ902111      | DQ902291 |          | Burbrink and Lawson (2007)                                  |
| <i>Pituophi deppei</i>            |               |          | AY122827 | Utiger <i>et al.</i> (2002)                                 |
| <i>Senticolis triaspis</i>        | DQ902127      | AF138775 | AY122848 | Burbrink and Lawson (2007); Utiger <i>et al.</i> (2002)     |

In the phylogenetic analyses, a low character to terminal taxa ratio (i.e., short sequences and many taxa) tended to result in low node supports, especially for the internal nodes (Kelly *et al.*, 2003). When the relatives of the three samples were found, their sequences should be grouped as “small datasets” to increase the character to taxa ratio, in order to further understand the relationship, particularly which species the three samples would belong to. Using RaxML v7.2.6, we estimated the ML tree and 1,000 nonparametric bootstraps (BS) using the GTR+I+G model for *cyt b* and ND4 combined sequences. The uncorrected *p*-distances within the “small datasets” were calculated using MEGA 4 (Tamura *et al.*, 2007).

### 3. Results

#### 3.1 Sequence characteristics Alignment by eye of the

*cyt b*, ND4 and 12s rRNA sequences of the three samples generated by this study resulted in 1015, 696, and 428 sites, respectively. The two neonate snakes (NS1 and NS2) shared identical haplotypes for *cyt b*, ND4 and 12s rRNA, respectively.

The percentages of the four nucleotides were relatively similar across Caenophidia, ratsnakes, *Elaphe*, and the new snakes, and showed strong bias against guanine, typical for mitochondrial DNA (de Queiroz *et al.* 2002; Malhotra and Thorpe, 2004; Huang *et al.* 2009; Table 2). While phylogenetic results (See below) suggest that the unidentified snakes are closely related to *Elaphe*, uncorrected *p*-distances among the three genes reveal that the new taxa attain the maximum difference within the genus (Table 3).

**3.2 Phylogenetic analyses** To find the systematic position of NSs and JS in Caenophidia, three datasets (*cyt b*,

**Table 2** Average nucleotide composition (%), numbers of variable (V) and parsimony informative (PI) sites for *cyt b*, ND4 and 12S rRNA gene fragments, based on the data used in the present study.

| Taxa              | Cyt <i>b</i> (1 015 aligned sites) |      |      |      |      |     | ND4 (696 aligned sites) |      |      |      |      |      | 12S rRNA(428 aligned sites) |            |      |      |      |      |      |     |            |
|-------------------|------------------------------------|------|------|------|------|-----|-------------------------|------|------|------|------|------|-----------------------------|------------|------|------|------|------|------|-----|------------|
|                   | T                                  | C    | A    | G    | V    | PI  | T                       | C    | A    | G    | V    | PI   | T                           | C          | A    | G    | V    | PI   |      |     |            |
| Caenophidia n=132 | 27.6                               | 29.8 | 31.6 | 11.0 | 718  | 645 | n=114                   | 25.5 | 29.6 | 33.4 | 11.5 | 471  | 414                         |            |      |      |      |      |      |     |            |
| Ratsnakes         | n=36                               | 28.0 | 28.1 | 32.9 | 11.0 | 491 | 407<br>40%              | n=36 | 26.7 | 27.6 | 34.4 | 11.3 | 355                         | 296<br>43% | n=33 | 20.4 | 24.5 | 37.8 | 17.3 | 140 | 100<br>23% |
| <i>Elaphe</i>     | n=9                                | 27.0 | 29.1 | 32.9 | 11.0 | 302 | 184                     | n=9  | 25.9 | 29.1 | 33.6 | 11.4 | 221                         | 140        | n=19 | 20.2 | 24.6 | 37.8 | 17.4 | 94  | 67         |
| NSs and JS        | n=2                                | 26.0 | 30.8 | 32.0 | 11.2 | 1   |                         | n=2  | 24.7 | 29.7 | 34.0 | 11.5 | 1                           |            | n=2  | 19.8 | 25.3 | 37.6 | 17.3 | 2   |            |

n: Numbers of sequences of each group.

%: PI/aligned sites

**Table 3** Sequence divergences between species of *Elaphe* for *cyt b*, ND4 and 12S rRNA sequences

| Taxa                     | Uncorrected <i>p</i> -distance |       |              |       |       |              |          |                    |              |
|--------------------------|--------------------------------|-------|--------------|-------|-------|--------------|----------|--------------------|--------------|
|                          | <i>cyt b</i>                   |       |              | ND4   |       |              | 12S rRNA |                    |              |
|                          | Max                            | Min   | Mean         | Max   | Min   | Mean         | Max      | Min                | Mean         |
| Ratsnakes                | 0.181                          | 0.067 | 0.138 (n=33) | 0.187 | 0.067 | 0.136 (n=33) | 0.139    | 0.039              | 0.086 (n=25) |
| <i>Elaphe</i>            | 0.132                          | 0.096 | 0.117 (n=7)  | 0.143 | 0.108 | 0.126 (n=7)  | 0.104    | 0.042 <sup>a</sup> | 0.068 (n=11) |
| NSs vs. JS               |                                |       | 0.001 (n=2)  |       |       | 0.002 (n=2)  |          |                    | 0.005 (n=2)  |
| NS/JS. vs. Ratsnakes     | 0.175                          | 0.107 | 0.142 (n=33) | 0.172 | 0.111 | 0.130 (n=33) | 0.111    | 0.067              | 0.085 (n=25) |
| NS/JS. vs. <i>Elaphe</i> | 0.142                          | 0.117 | 0.129 (n=8)  | 0.140 | 0.127 | 0.132 (n=8)  | 0.104    | 0.069              | 0.088 (n=11) |

<sup>a</sup>: Despite the distance of 0.005 between *E. anomala* and *E. schrenckii*, they may be conspecific, or the former is a subspecies of the latter (Pope, 1935; Utiger *et al.*, 2002).

n: Numbers of sequences of each group.

ND4, and *cyt b* +ND4) from Huang *et al.* (2009) were used with BI and ML approaches. These topologies were similar to those presented in Huang *et al.* (2009). The basic relationships of the commonly recognized families and/or subfamilies are similar: *Acrochordus* (Viperidae (Atractaspidinae, Boodontinae, Elapinae, Hydrophiinae, Psammophiinae, Pseudoxyrhophiinae), (Colubrinae, Natricinae, Xenodontinae)). This basic relationship is the same as that of Kelly *et al.* (2003) and Lawson *et al.* (2005). In our trees, NS1, NS2 and JS fell within the ratsnakes (including the Old World genera *Coronella*, *Elaphe*, *Euprepophis*, *Oocatochus*, *Oreophis*, *Orthriophis*, *Rhinechis*, *Zamenis*, and New World genera *Bogertophis*, *Lampropeltis*, *Arizona*, *Pseudelaphe*, *Pantherophis*, *Senticolis*; Utiger *et al.*, 2002; Burbrink and Lawson, 2007) with high (BI trees) and moderate (RAxML trees) supports (Table 4).

The topologies of the ratsnakes were similar among the individual mtDNA genes and their combination, except with respect to the uncertainty of the monophyly of the genus *Orthriophis*. The three samples (NS1, NS2 and JS) are placed, with high (PP) or moderate (BS) support, as sister to *Elaphe* (Table 4). Due to the difficulty in aligning 12S rRNA, the sequences were found to yield trees with poor resolution.

#### 4. Discussion

Comprehending the diversity of snakes within any region has traditionally relied on the analyses of morphological data. However, for many species of snakes, morphological data have proven difficult to uncover cryptic diversity and to understand the relationships among taxa, due in part to their specialized and simple body structures (Huang *et al.* 2009; Burbrink and Crother, 2010). Therefore, understanding the relationships among snakes glob-

ally and regionally has excelled in the last decade due to molecular phylogenetic methods (Lawson *et al.*, 2005; Huang *et al.*, 2009; Burbrink and Crother, 2010).

Utiger *et al.* (2002) examined the phylogenetic relationships of the Holarctic ratsnakes (*Elaphe* auct.) using portions of sequences from two mitochondrial genes, 12s rRNA and CO1. They proposed some taxonomic changes, established two new genera (*Orthriophis* and *Oreophis*, gen. nov.), and concluded that *Elaphe* (sensu stricto) Fitzinger, 1833 is made up of ten Palearctic species: *E. sauromates* (type species), *E. anomala*, *E. bimaculata*, *E. carinata*, *E. climacophora*, *E. davidi*, *E. dione*, *E. quadrivirgata*, *E. quatuorlineata*, and *E. schrenckii*. Their conclusions are generally verified in another study using more data and taxa (Burbrink and Lawson, 2007).

The three enigmatic samples in the present study always form a monophyletic clade and are closely related to *Elaphe*, and possibly are the sister species to the remaining members of the genus (Figure 1, Table 4). The very small genetic distance among the three specimens (0.001 for *cyt b*, 0.002 for ND4, 0.005 for 12s rRNA, see Table 3) suggest they are part of the same population. Additionally, the minimum genetic distance between these samples and *Elaphe* is always greater than that among the species within this genus: 0.117>0.096 for *cyt b*, 0.127>0.108 for ND4, and 0.069>0.042 for 12s rRNA (Table 3).

We conclude that these specimens likely represent a yet undescribed species of *Elaphe*, elevating the number of species in this genus to 11 and the number of total ratsnakes in China to 16. This would also elevate the number of snakes in the Zoige to 3. We anticipate acquiring additional material to formally describe this new taxon, characterize its mensural and meristic, and morphological variation, estimate its distribution range in western China, and describe its general ecology.

**Table 4** Support of the monophyly of *Elaphe* (including NSs and JS) and the ratsnakes in phylogenetic trees based on various datasets

|                 | Ratsnakes in Caenophidia |             |                   | <i>Elaphe</i> within the ratsnakes |            |                   |
|-----------------|--------------------------|-------------|-------------------|------------------------------------|------------|-------------------|
|                 | Cyt b (n=132)            | ND4 (n=114) | Cytb + ND4 (n=87) | Cyt b (n=36)                       | ND4 (n=36) | Cytb + ND4 (n=36) |
| Posterior Prob. | 98                       | 100         | 98                | 100                                | 74         | 100               |
| Bootstraps      | 68                       | —           | 75                | 76                                 | —          | 85                |

n: Numbers of sequences of each dataset.

—: < 50.

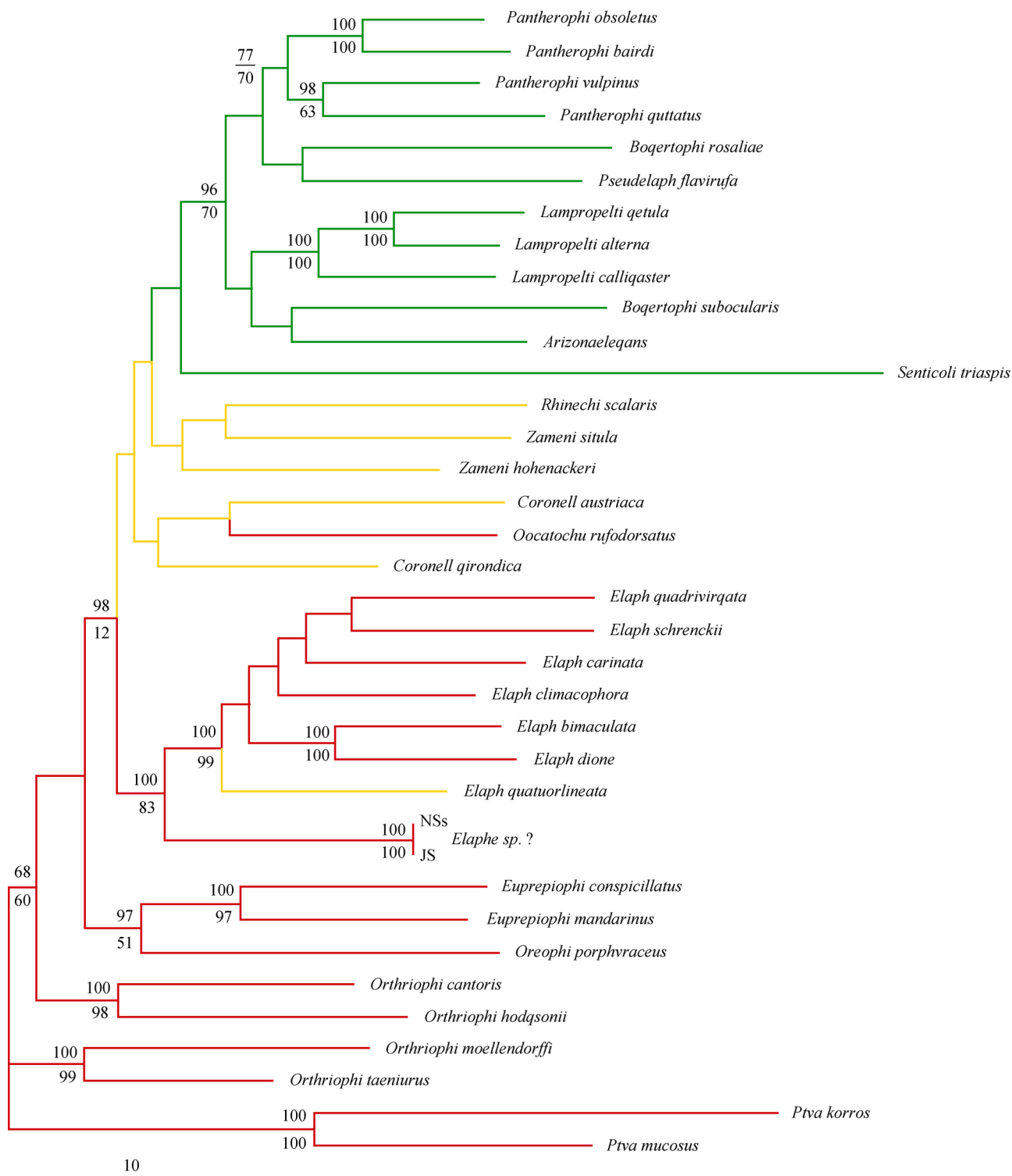


**Figure 1** The 50% majority rule consensus tree from the Bayesian analysis based on *cyt b* and ND4 combined sequences. Posterior probability values and RAxML supports are shown above and below the corresponding nodes (under 50% from both analyses omitted). Likelihood settings from best-fit model (GTR+I+G) selected by BIC in Modeltest 3.7 ( $-\ln L=19544.7363$ ). Base= (0.3799 0.3230 0.0781), Nst=6, Rmat=(0.7682 9.9929 1.4101 0.3322 20.6632), Rates=gamma, Shape=0.6621, Pinvar=0.4522. Red: Mainly eastern Palearctic or Oriental distribution; Yellow: Mainly western Palearctic distribution; Green: Mainly New World distribution (according to Utiger *et al.*, 2002).

**Acknowledgments** This work is supported by the National Natural Science Foundation of China (30870290) and the Science Research Program of Huangshan University (2006xkj014). We thank WAN Tao, SUN Xiaoyu, ZHANG Baolin and CHEN Jinmin (Huangshan University) for their help in data processing.

## References

- Arévalo E., Davis S. K., Sites J. W., Jr. 1994. Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the *Sceloporus grammicus* complex (Phrynosomatidae) in Central Mexico. *Syst Biol*, 43: 387–418
- Burbrink F. T., Lawson R., Slowinski J. B. 2000. Mitochondrial DNA phylogeography of the polytypic North American rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. *Evolution*, 54: 2107–2118
- Burbrink F. T., Lawson R. 2007. How and when did Old World ratsnakes disperse into the New World? *Mol Phyl Evol*, 43: 173–189
- Burbrink F. T., Crother B. I. 2010. Evolution and taxonomy of snakes. In *Reproductive Biology and Phylogeny of Snakes* (Aldridge and Sever, eds). New Hampshire: Science Publishers, Inc (In review/press)
- Burbrink F. T., Pyron R. A. 2010. How does ecological opportunity influence rates of speciation, extinction, and morphological diversification in New World ratsnakes (Tribe Lamprodelphini)? *Evolution*, 64: 934–943
- de Queiroz A., Lawson R., Lemos-Espinal J. A. 2002. Phylogenetic relationships of North American garter snakes (*Thamnophis*) based on four mitochondrial genes: How much DNA sequence is enough? *Mol Phyl Evol*, 22: 315–329
- Huang S., Liu S. Y., Guo P., Zhang Y. P., Zhao E. M. 2009. What are the closest relatives of the hot-spring snakes (Colubridae, *Thermophis*), the relict species endemic to the Tibetan Plateau? *Mol Phyl Evol*, 51: 438–446
- Huelsenbeck J. P., Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17: 754–755
- Kelly C. M. R., Barker N. P., Villet M. H. 2003. Phylogenetics of advanced snakes (Caenophidia) based on four mitochondrial genes. *Syst Biol*, 52: 439–459
- Lawson R., Slowinski J. B., Crother B. I., Burbrink F. T. 2005. Phylogeny of Colubroidea (Serpentes): New evidence from mitochondrial and nuclear genes. *Mol Phyl Evol*, 37: 581–601
- Malhotra A., Thorpe R. S. 2004. A phylogeny of four mitochondrial gene regions suggests a revised taxonomy for Asian pit-vipers (*Trimeresurus* and *Ovophis*). *Mol Phyl Evol*, 32: 83–100
- Nagy Z. T., Lawson R., Joger U., Wink M. 2004. Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers. *J Zool Syst Evol Res*, 42: 223–233
- Pope C. H. 1935. The Reptiles of China. Turtles, Crocodilians, Snakes, Lizards. New York: The American Museum of Natural History, Natural History of Central Asia, V: 266–271
- Posada D., Crandall K. A. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics*, 14: 817–818
- Pyron R. A., Burbrink F. T. 2009. Lineage diversification in a widespread species: Roles for niche divergence and conservatism in the common kingsnake, *Lampropeltis getula*. *Mol Ecol*, 18: 3443–3457
- Sambrook J., Fritsch E. F., Maniatis T. 1989. Molecular cloning: A laboratory manual, second ed., New York: Cold Spring Harbor Laboratory Press
- Schulz K. D. 1996. A monograph of the colubrid snakes of the genus *Elaphe* Fitzinger. Havlickuv Brod, Czech Republic: Koeltz Scientific Books
- Stamatakis A., Hoover P., Rougemont J. 2008. A rapid bootstrap algorithm for the RAxML Web-Servers. *Syst Biol*, 57: 758–771
- Tamura K., Dudley J., Nei M., Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol*, 24: 1596–1599
- Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. 1997. The CLUSTAL\_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res*, 25: 4876–4882
- Ursenbacher S., Conelli A., Golay P., Monney J. C., Zuffi M. A., Thiery G., Durand T., Fumagalli L. 2006. Phylogeography of the asp viper (*Vipera aspis*) inferred from mitochondrial DNA sequence data: Evidence for multiple Mediterranean refugial areas. *Mol Phyl Evol*, 38: 546–552
- Utiger U., Helfenberger N., Schätti B., Schätti C., Ruf M., Ziswiler V. 2002. Molecular systematics and phylogeny of Old World and New World ratsnakes, *Elaphe* Auct., and related genera (Reptilia, Squamata, Colubridae). *Russ J Herpetol*, 9: 105–124
- Zaher H., Grazziotin F. G., Cadle J. E., Murphy R. W., de Moura-Leite J. C. D., Bonatto S. L. 2009. Molecular phylogeny of advanced snakes (Serpentes, Caenophidia) with an emphasis on South American Xenodontines: A revised classification and descriptions of new taxa. *Pap Avulsos Zool*, 49: 115–153
- Zhao E. M. 2003. Coloured Atlas of Sichuan Reptiles. Beijing: Forestry Publishing House, 292 pp (In Chinese)
- Zhao E. M. 2006. Snakes in China, Vol. 1, Hefei: Anhui Science and Technology Publishing House (In Chinese)